OPEN PEER REVIEW

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Peer Review Report for "Analgesic Effects of Formononetin: An Investigation of the Role of Hypothalamic Corticotrophin Releasing Hormone, Orexin, and Melanin Concentrating Hormone Signaling Pathways in a Pain Model of Rats"

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Review Timeline:

Submit Date:	7 Aug 2024
Revised Date:	22 Dec 2024
Accepted Date:	25 Dec 2024

Revision (0)

Here, you can see the **Reviewers**, **Associate Editors** and **EICs'** comments from the beginning to the end of the revision process.

REFEREE : Reviewer

9 Sep 2024

The manuscript investigates the effects of formononetin, a phytoestrogen with potential analgesic properties, on the gene expression of orexin, Crh, and Mch in Wistar rats subjected to pain induction via formalin injection.

Weaknesses:
1. Limited Novelty: The study primarily presents conventional experimental approaches and does not offer significant innovation or insight beyond established knowledge in the field. The findings are somewhat predictable given the existing literature on phytoestrogens and pain modulation.
2. Insufficient Depth: While the study provides data on mRNA levels, it lacks

comprehensive analysis regarding the broader implications of these findings. Moreover, the connection between gene expression changes and behavioral outcomes could be more explicitly articulated.
3. Sample Size and Control: The use of a small sample size (n=5 per group) may limit the reliability and generalizability of the findings. Additionally, further control for potential confounding variables in the pain model would strengthen the study.

While the study addresses an important area of research and presents foundational data, the overall contribution to the field is limited by its conventional approach and lack of depth. Therefore, I recommend reconsideration of the analysis, depth, and innovative aspects before reconsideration for publication in a peer-reviewed journal.

Sara Bahrainian : Associate Editor

11 Sep 2024

Thank you for submitting your manuscript to Jundishapur Journal of Natural Pharmaceutical Products.
Please revise the manuscript and submit a point-by-point response to the reviewers' comments and concerns.

I would also greatly appreciate the authors' contribution and time for reviewing manuscripts for this journal. Without the dedication of reviewers like you, it would be impossible to manage an efficient peer review process and maintain the high standards necessary for a successful journal.

OPEN PEER REVIEW

Revision (1)

Reply to Reviewers

Ideally, the reviewing process can significantly improve the submitted manuscripts by allowing the authors to take into account the advice of reviewers. Author(s) must reply to all reviewers' comments in a separate Word file, point by point. A "**Reply to Reviewers**" document is submitted along with revised manuscript during submission of revised files, summarizing the changes that the authors made in response to the reviewers' comments. The responses to reviewers' comments specifies how the authors addressed each comment the reviewers made.

You can read the authors' responses to the reviewers' comments in the next page.

Dear Editor in- Chief

By special thanks of valuable comments of dear reviewers to improve the quality of the present manuscript, the responses were highlighted in the text. Also they were mentioned in the present file as following:

Response to Reviewer1

1. Limited Novelty: The study primarily presents conventional experimental approaches and does not offer significant innovation or insight beyond established knowledge in the field. The findings are somewhat predictable given the existing literature on phytoestrogens and pain modulation.

The present study is a basic research and it aims to find the molecular mechanisms of analgesic effects of formononetin. So, based on dear reviewer guidance the following part was added to the text:

Current chemical analgesic medicines have significant adverse effects, including gastrointestinal issues, emesis, tolerance, or addiction. Therefore, to modulate pain, new medicines derived from plants, as a complementary or alternative approach, are needed to reduce adverse effects. Formononetin is a phytostrogen derivative of *Red clover* which is consumed to reduce pain and hot flash based on traditional medicine. Formononetin exhibits significant estrogenic capabilities, antioxidant and anti-inflammatory properties and previous studies documented its analgesic effects based on behavioral tests. However, the molecular mechanisms mediating the analgesic effects of formononetin are still unclear. So novelty of the present study may be finding the central mechanisms through the formononetin may mediate the analgesic effects.

2. Insufficient Depth: While the study provides data on mRNA levels, it lacks comprehensive analysis regarding the broader implications of these findings. Moreover, the connection between gene expression changes and behavioral outcomes could be more explicitly articulated.

As mentioned in the discussion and results section, there is a close relationship between pain behaviors and the expression of genes related to pain. It was observed by the behavioral test that formononetin reduces the pain score. Gene expression evaluation showed that formononetin may modulate the pain-related genes.

3. Sample Size and Control: The use of a small sample size (n=5 per group) may limit the reliability and generalizability of the findings. Additionally, further control for potential confounding variables in the pain model would strengthen the study.

We really appreciate valuable comment of dear reviewer. But that's necessary to mention that based on the previous studies and the law of ethic committee for experimental studies, the sample size of animals for in vivo studies often is chosen as n=5 per group. So, we have to some limitation to choose sample size of animals for experimental research studies.

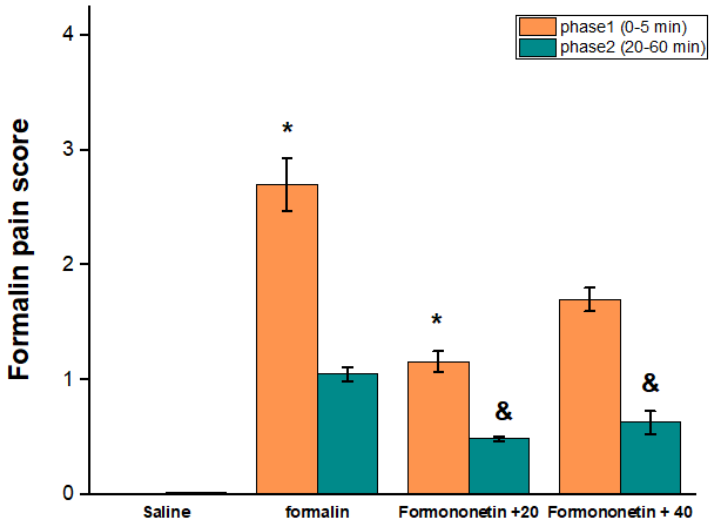
Response to Reviewer2

1. Can you clarify the rationale for the chosen doses of formononetin (20 µg and 40 µg) in your experiments? How were these doses determined? Can you elaborate on the selection criteria for the dosage of formononetin (20 µg and 40 µg) used in this study? Were these doses based on prior studies, and if so, how were they determined to be appropriate for this model?

To select the effective doses of drug, we used behavioral tests related to pain responses. So, at first, rats in several groups received different doses of the drug and between the used doses, based on behavioral responses we choose 20 and 40 micrograms formononetin to investigate the molecular mechanisms may mediate the anti- pain effects of formononetin.

2. What controls were implemented to ensure the reliability and reproducibility of the behavioral tests used in your study?

The study used a control group that received saline. But they were not included in the figure due to not showing noticeable behavior. At the request of the respected reviewer, the control group was added to the figure



3. How do you account for the variability in gene expression results among individual animals within the same treatment group?

To reduce the effect of individual variability in gene expression results among individual animals within the same treatment group, a housekeeping gene, GAPDH used to normalize the gene expression of each sample.

4. Can you provide more details on the statistical analyses used to assess the significance of your findings? Were any adjustments made for multiple comparisons?

For analysis the data related to the behavioural results, gene expression levels of orexin, Crh, and Mch, we used separate one-way ANOVA test. Also for determining the significant comparison between groups Tukey's post hoc test was performed following each one-way ANOVA test.

5. You mention that formononetin may downregulate orexin and Crh gene expression. Can you elaborate on the specific molecular pathways involved in this regulation?

The molecular mechanisms of formononetin have not been investigated in previous studies. In our recent work, the intra molecular mechanisms of the analgesic effects of formononetin was investigated for first time. There is no previous study on specific molecular mechanisms of formononitin. Few studies have been studied the molecular mechanism of formononitin effects on physiological action, however several studies have been confirmed its using via behavioural tests, antioxidant and anti-inflammatory effects. So, it makes difficult to compare the present intra hypothalamic molecular mechanisms of analgesic effects of formononetin with previous ones. As dear reviewer guidance, some molecular pathways involved in downregulating of orexin and Crh gene expression by formononetin was added to text as following:

Formononetin may downregulate orexin and Crh gene expression via interaction with GABAergic and glutamatergic system. Inputs from GABAergic neurons in the lateral hypothalamus regulate orexin activity. Also the GABAergic neurons regulate orexin neurons through an inhibitory mechanism. Formononetin has been proven to have a regulatory effect on the GABAergic and glutamatergic system. A previous study highlights the glutamate's role as a primary excitatory neurotransmitter in the hypothalamus, particularly influencing the orexin neurons. Glutamate could impact orexin neuron activities via NMDA and non-NMDA receptors. Additionally, synaptic connections of glutamatergic axon terminals were documented on orexin neurons. On the other hand, formononetin is able to protect neurons against glutamate induced excitotoxic damage. So, formononetin may participate in down regulation of the *orexin* gene expression due to its anti-glutamatergic impacts.

Also, another mechanism of formononetin to downregulate orexin and Crh gene expression may be due to its effects on α1-adrenoceptors. A study demonstrated that formononetin reduces the expression of α1-adrenoceptors receptors. The α_1-adrenoceptors co-localize with nociceptive markers in dorsal root ganglia. The expression of α_1-adrenoceptors on C- and A-delta nociceptive afferent fibres provides a histochemical substrate for direct excitation of these fibres by adrenergic agonists. Also, a study showed that α_1-adrenoceptors expression increases on orexin A and CRH neurons during painful conditions. On the other hand, evidence shows that CRH and orexin levels increase in pain. Therefore, it is assumed that formononitin, leads to a decrease in *Crh* and *orexin* gene expression by reducing the activity of α_1-adrenoceptors in pain model of rats.

Zheng H, Lim JY, Seong JY, Hwang SW. The role of corticotropin-releasing hormone at peripheral nociceptors: implications for pain modulation. Biomedicines. 2020 Dec 17;8(12):623.

Suemaru S, Dallman MF, Darlington DN, Cascio CS, Shinsako J. Role of alpha-adrenergic mechanism in effects of morphine on the hypothalamo-pituitary-adrenocortical and cardiovascular systems in the rat. Neuroendocrinology. 1989 Apr 2;49(2):181-90.

Stone EA, Lin Y, Ahsan MR, Quartermain D. Evidence of roles of central α1-adrenoceptors and epinephrine in orexin A-induced hyperactivity in mice. Neuroscience letters. 2005 Jun 24;381(3):325-8.

Dawson LF, Phillips JK, Finch PM, Inglis JJ, Drummond PD. Expression of α1-adrenoceptors on peripheral nociceptive neurons. Neuroscience. 2011 Feb 17;175:300-14.

Sun, T., Wang, J., Huang, L.H. and Cao, Y.X., 2013. Antihypertensive effect of formononetin through regulating the expressions of eNOS, 5-HT2A/1B receptors and α1-adrenoceptors in spontaneously rat arteries. *European Journal of Pharmacology*, *699*(1-3), pp.241-249.

6. How do you hypothesize that formononetin interacts with the GABAergic and glutamatergic systems to produce its effects on pain modulation?

This hypothesis has been suggested based on the results of the previous studies which have reported the interaction of formononetin with GABAergic and glutamatergic systems. Some of related references are mentioned as following:

Tian Z, Liu Sb, Wang Yc, Li Xq, Zheng Lh, Zhao Mg. Neuroprotective effects of formononetin against NMDA-induced apoptosis in cortical neurons. *Phytotherapy Research*. 2013;**27**(12):1770-5. https://doi.org/ 10.1002/ptr.4928.

Wang X-s, Guan S-y, Liu A, Yue J, Hu L-n, Zhang K, et al. Anxiolytic effects of Formononetin in an inflammatory pain mouse model. *Molecular brain*. 2019;**12**:1-12. https://doi.org/10.1186/s13041-019-0453-4

7. How do the effects of formononetin compare to other known analgesics in terms of efficacy and mechanism of action?

The most important drugs used as pain relievers include opioids and non-steroidal anti-inflammatory drugs (NSAIDs) which both groups have serious side effects. The NSAIDs often cause side effects, especially in the upper gastrointestinal tract and cardiovascular system and opioids cause drug abuse and dependence. So, finding natural painkillers are interest of recent studies to reduce side effects and complication of these drugs. phytoestrogens, as natural compounds show anti-stress and anti-pain effects. However, exact molecular mechanisms mediating their analgesic effects are not clear. The results of present study demonstrated that formononetin may able to affect both phase of formalin induced paid and alteration of of hypothalamic CRH, orexin and MCH neural pathways may be partly associated with its pain killing effect. The action and pain relieving mechanism of formononetin may be more similar to opioid than NAAIDs. As previous studies have shown that diclofenac and ibuprofen as NSAIDs only affect inflammatory phase of formalin induced pain and their main mechanism for pain killing is due to inhibition of cyclooxygenase enzyme and prostaglandin synthesis. However, morphine and other opioid mainly act as central acting analgesics and exert both central and peripheral effects. It has been established that morphine exerts anti-nociceptive effects on both phase of formalin induced pain and hypothalamic neural pathways are associated with its analgesic effects.

De Paz-Campos MA, Ortiz MI, Piña AE, Zazueta-Beltrán L, Castañeda-Hernández G. Synergistic effect of the interaction between curcumin and diclofenac on the formalin test in rats. Phytomedicine. 2014 Oct 15;21(12):1543-8.

McDaid C, Maund E, Rice S, Wright K, Jenkins B, Woolacott N. Paracetamol and selective and non-selective non-steroidal anti-inflammatory drugs (NSAIDs) for the reduction of morphine-related side effects after major surgery: a systematic review. Health Technol Assess. 2010 Mar 1;14(17):1-53.

Dutra JM, Espitia PJ, Batista RA. Formononetin: Biological effects and uses–A review. Food chemistry. 2021 Oct 15;359: 129975.

B H Manning [1], M J Morgan, K B Franklin. Morphine analgesia in the formalin test: evidence for forebrain and midbrain sites of action Neuroscience. 1994 Nov;63(1):289-94.

8. Have you considered the potential impact of other phytoestrogens on pain modulation, and how do they compare to formononetin in this context?

The present study aimed to investigate the molecular mechanisms mediating the analgesic effects of formononetin. However, several previous studies have investigated the analgesic effects of other phytostrogens including genistein, resveratrol and daidzein. Based on dear reviewer guidance, following part and related references were added to the discussion to compare the potential impact of other phytoestrogens on pain modulation with formononetin.

Genistein, resveratrol and daidzein, a natural phytoestrogen, is structurally similar to E2 and can directly bind to estrogen receptors (ERs) to regulate E2 signalling pathway. The mechanism of analgesic effects of phytoestrogens is through inhibition of inflammation. Their analgesic effects have been investigated in several studies. Genistein reduces neuropathic pain caused by peripheral nerve damage. Also, genistein has positive effects on histopathology, inflammation and peripheral nerve damage. Daidzein reduced paclitaxel-induced neuropathic pain sensitivity. Administration of daidzein led to inhibition of neuroinflammation through increasing antioxidant enzymes and reducing oxidative stress markers. daidzein also inhibited pro-inflammatory mediators. The analgesic effect of resveratrol was proved in the formalin induced pain. Resveratrol acts on various pathways related to pain perception and transmission, such as suppressing inflammatory mediator production through inhibiting NF-B, as well as inhibiting cyclooxygenase enzymes. Additionally, it has a potent inhibitory effect on transient receptor potential cation channel, subfamily A member 1 (TRPA1) and transient receptor potential cation channel, subfamily V member 1 (TRPV1), channels involved in nociceptive signalling.

Özbek Z, Aydin H, Kocman A, Özkara E, Şahin E, Bektur E, Vural M, Köse A, Arslantaş A, Baycu C. Neuroprotective effect of genistein in peripheral nerve injury. Turkish neurosurgery. 2017;27(5).

Sajjad Q, Khan AU, Khan A. Pharmacological investigation of genistein for its therapeutic potential against nitroglycerin-induced migraine headache. Journal of Pharmacy and Pharmacology. 2024 Jul 15:rgae084.

Zafar S, Luo Y, Zhang L, Li CH, Khan A, Khan MI, Shah K, Seo EK, Wang F, Khan S. Daidzein attenuated paclitaxel-induced neuropathic pain via the down-regulation of TRPV1/P2Y and up-regulation of Nrf2/HO-1 signaling. Inflammopharmacology. 2023 Aug;31(4):1977-92.

Rojas-Aguilar FA, Briones-Aranda A, Jaramillo-Morales OA, Romero-Nava R, Esquinca-Avilés HA, Espinosa-Juárez JV. The Additive Antinociceptive Effect of Resveratrol and Ketorolac in the Formalin Test in Mice. Pharmaceuticals. 2023 Jul 28;16(8):1078.

9. What implications do your findings have for the potential therapeutic use of formononetin in clinical settings for pain management?

The present study is a basic research and it aims to find the molecular mechanisms of analgesic effects of formononetin which is used traditionally to reduce pain and hot flash. Based on the present results the involvement of CRH and orexin signaling pathways in mediating the effects of formononetin on reducing pain may suggest its potential therapeutic use for management stress too. However, further studies are needed to investigate the effects of formononetin on stress through the CRH and orexin signaling pathways.

10. Are there any known side effects or contraindications associated with formononetin that should be addressed in future studies?

People with conditions such as breast cancer, endometriosis, or uterine fibroids may need to avoid formononetin due to its estrogenic activity. The safety of formonontin during pregnancy and breast-feeding has not been well established, so caution is advised.

11. How does your work build on or differ from previous studies regarding the analgesic effects of formononetin or similar compounds?

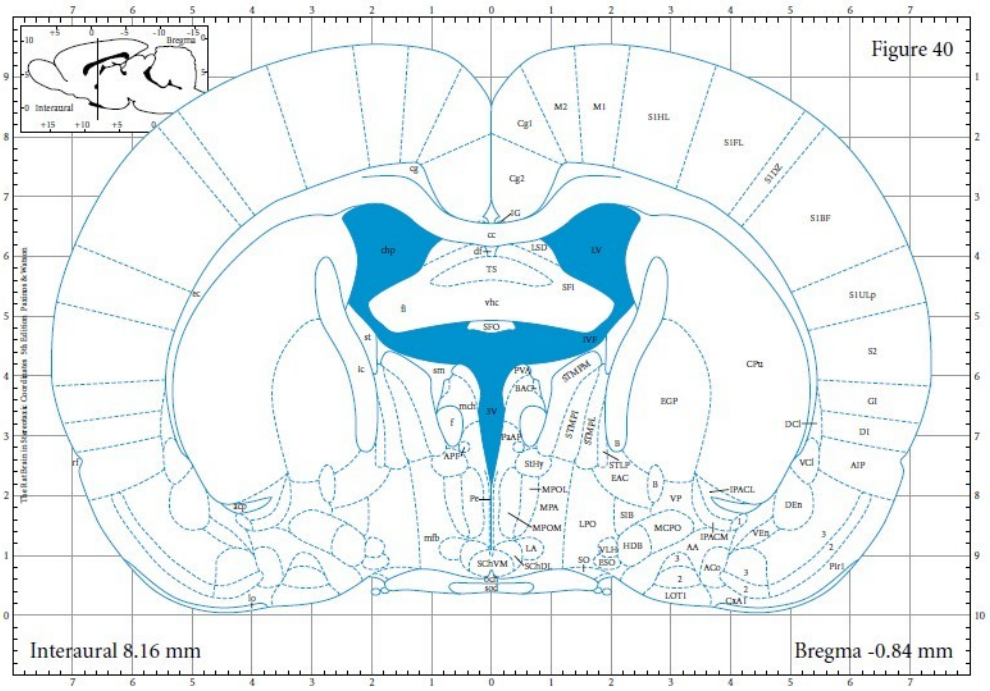
The present behavioral results are according to the previous studies which showed the analgesic effects of formononetin in behavioural tests. However, the molecular mechanisms mediating the analgesic effects of formononetin was investigated for the first time in the present study and there is not previous studies about the intra-hypothalamic neuropeptides involved in pain management.

12. Can you discuss any limitations of the existing literature that your study aims to address?

The present study, has been focused on acute pain and determination of mRNA changes due to management of research expense. For further studies it is suggested chronic pain and measurement of protein levels by using western blot technique.

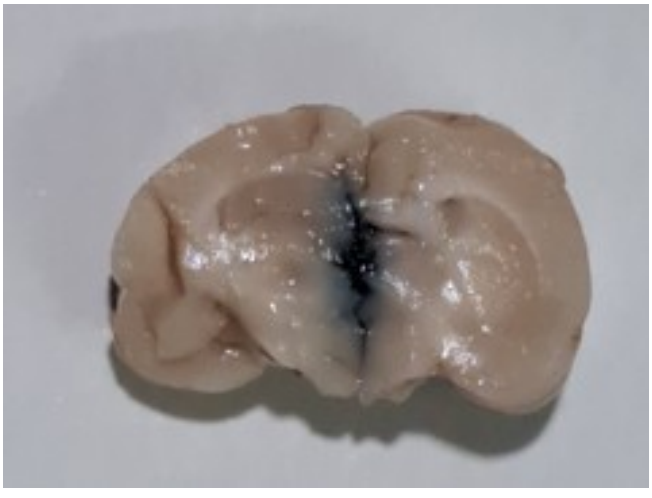
13. Could you provide more details on the stereotaxic surgery procedure? How was the accuracy of cannula placement ensured, and what measures were taken to minimize variability?

The animal was placed in a stereotaxic apparatus to maintain a stable position. The head was aligned using ear bars so that the skull conformed to standard anatomical landmarks. A midline scalp incision was made to expose the skull. Third cerebral ventricle coordinates were chosen based on the information of Watson and Paxinos atlas. The special cannula was fixed on the skull at coordinates of third cerebral ventricle. At the end of study, the right location of cannula was confirmed by providing brain samples. To see the cannulation site more clearly, Evans Blue dye was injected before cutting brain slices. The figure related to the third cerebral ventricle coordinates based on Watson and Paxinos atlas and figures provided by cutting brain slices are shown as following:





8



14. How did you ensure that the behavioral observations were unbiased? Were the observers blinded to the treatment groups during the behavioral tests?

Observers conducting the behavioural tests were blinded to the treatment groups. A person was done the injection of drugs and other person was recorded the behavioural tests. This means they did not know which animals received which treatment, preventing any preconceived notions from influencing their assessments. Animals were randomly assigned to treatment groups prior to testing. This randomization helps ensure that any potential biases in animal characteristics are evenly distributed across groups. Also, behavioural tests recorded by a camera to further accurate analysis.

15. Can you clarify the selection of GAPDH as a reference gene for RT-PCR? Were other reference genes considered, and how was their stability assessed in your experimental conditions?

Based on several related article in different journals, GAPDH was chosen as a reference gene for RT-PCR. In fact to reduce the effect of individual variability in gene expression results among individual animals within the same treatment group, a housekeeping gene, GAPDH used to normalize the gene expression of each sample.

16. The results section mentions a significant decrease in pain scores for both phases in the 20 µg formononetin group. Could you provide more context on the clinical relevance of these findings?

The advantage of the formalin-pain test of nociception is that it can determine pain in its central and peripheral components. The model includes of the two different phases which can be separated in time: The early phase, which starts immediately following injection of formalin, and is probably due to direct chemical stimulation of nociceptors (acute pain). second phase starts approximately 15 min following formalin injection and inflammatory processes via affecting central signalling pathways are involved in mediating this phase. Central analgesic drugs, inhibited equally in both phases, whereas peripherally acting drugs, including steroids and NSAIDs inhibited mainly pain in the later phase. Our data showed that formononetin may able to act as central analgesic drugs to affect both phase 1 and also phase 2 due to its anti-inflammatory properties.

17. In your discussion, you mention the potential mechanisms through which formononetin exerts its effects. Can you provide more detailed insights into how these mechanisms were inferred from your results?

To suggest some potential mechanisms by which mRNA levels of CRH, orexin or MCH may be affected by formononetin, we try to collected the all previous data regarding to formononetin to present some possible mechanism which may be involved in altering CRH, orexin or MCH by formononetin. The potential mechanisms through which formononetin exerts its effects are summarized as following:

Formononetin can bind to estrogen receptors (ERα and ERβ), mimicking the effects of estrogen. This interaction may influence pain pathways and reduce inflammation, as estrogen has been shown to modulate pain perception. Formononetin may inhibit pro-inflammatory cytokines and mediators, leading to a reduction in inflammation. By decreasing inflammation, it could alleviate pain associated with inflammatory conditions. Formononetin might influence the release or uptake of neurotransmitters involved in the pain signalling pathways, such as serotonin and norepinephrine, potentially altering pain perception. Formononetin has antioxidant properties that may help reduce the oxidative stress in tissues. By mitigating oxidative damage, it could contribute to pain relief, especially in conditions where oxidative stress is a contributing factor. Research suggests that formononetin may affect various signalling pathways involved in pain transmission, including the modulation of ion channels and receptors that play a role in nociception (the sensory perception of pain). Formononetin may influence the expression of genes related to pain and inflammation, further contributing to its analgesic effects. These mechanisms highlight the multifaceted approach through which formononetin may exert its analgesic properties, making it a promising candidate for further research in pain management.

18. Based on your findings, what are the potential clinical implications of using formononetin for pain management? Are there any limitations or considerations that need to be addressed before moving to clinical trials?

The potential clinical implications of using formononetin for pain management are promising, considering its multifaceted mechanisms of action and its phytoestrogenic properties. However, several considerations and limitations should be addressed before moving to clinical trials. Formononetin can act as a natural alternative to conventional painkillers, potentially reducing opioid dependence and minimizing associated side effects. Its ability to modulate estrogen receptors may provide targeted pain relief for conditions

affected by hormonal fluctuations, such as menstrual pain or menopausal symptoms. The anti-inflammatory effects of formononetin can be useful in the treatment of chronic pain conditions characterized by inflammation, such as arthritis or fibromyalgia. If proven effective, formononetin may also have neuroprotective properties, potentially beneficial for patients with neuropathic pain. Formononetin can be considered as part of combination therapies, increasing the effectiveness of existing treatments while reducing doses and side effects. Most current findings are based on preclinical studies, and robust clinical trials are necessary to determine efficacy, safety, optimal dosing, and potential side effects in humans. Individual responses to phytoestrogens can vary significantly due to hormonal status, and existing medical conditions. Understanding these variables is important for personalized treatment approaches. The bioavailability of formononetin and its metabolites needs to be studied to determine effective dosage regimens. Factors affecting absorption and metabolism should also be considered. As a natural combination, navigating regulatory pathways for clinical trials may present challenges. Ethical considerations regarding informed consent and potential risks must also be addressed. The long-term effects of the use of formononetin need to be investigated to ensure that chronic use does not lead to unwanted consequences.

• From linguistic, grammatical, and structural aspects:

A) The phrase "the pain model group, which received saline" could be clearer if rephrased as "the pain model group that received saline." The use of "which" can sometimes lead to ambiguity; "that" is more precise for defining clauses.

According to the reviewer's opinion, the grouping was corrected as following:

groups 1 and 2, respectively, had been the control group and the pain model group, that received saline. Groups 3 and 4 have been the pain model rats that received 20 and 40 µg of formononetin.

B) The term "phytoestrogen" is used appropriately, but it may require a brief definition for clarity, especially for readers unfamiliar with the term.

Phytoestrogens are natural compounds that exist in many plants and foods. These compounds have a similar chemical structure to estrogen.

C) "Previous studies has been shown that formononetin exhibits significant estrogenic capabilities" should be corrected to "Previous studies have shown that formononetin exhibits significant estrogenic capabilities.

Based on dear reviewer's guidance, the proposed sentence was replaced as following:
Previous studies have shown that formononetin exhibits significant estrogenic capabilities.

D) "The results of Jang et al. showed that MCH plays an important role in pain modulation and the administration of MCH showed analgesic effects in the hot plate test" could benefit from a comma before "and." Proper punctuation helps in breaking down complex sentences for better readability.
Based on dear reviewer's guidance, this item was edited.

E) Some paragraphs, particularly in the background section, are lengthy and could be broken down into smaller paragraphs for better readability.
Based on dear reviewer's guidance, the text was structurally rechecked.

• Another examples:
Example: "One of the isoflavonoid groups of phytoestrogens, formononetin, is a chemical found in many leguminous plants and Red clover."
• Error: The phrase "one of the isoflavonoid groups" is awkward.
• Correction: "Formononetin is an isoflavonoid found in many leguminous plants and red clover."
Based on dear reviewer's guidance, the proposed sentence was replaced as following:
Formononetin is an isoflavonoid found in many leguminous plants and red clover.

Example: "The physiological function of orexin in pain modulation has been confirmed by studding the mechanical, chemical, thermal, and nociceptin-induced pain."
• Error: "Studying" is misspelled as "studding."
• Correction: "The physiological function of orexin in pain modulation has been confirmed by studying mechanical, chemical, thermal, and nociceptin-induced pain."
Based on dear reviewer's guidance, the proposed sentence was replaced as following:
The physiological function of orexin in pain modulation has been confirmed by studying the mechanical, chemical, thermal, and nociception-induced pain.

Example: "Corticotrophin releasing hormone (CRH) is one of the most crucial hormones in regulating the hypothalamic-pituitary-adrenal (HPA) axis."
• Error: The phrase is overly complex.

• Correction: "Corticotrophin-releasing hormone (CRH) plays a key role in regulating the hypothalamic-pituitary-adrenal (HPA) axis."

Based on dear reviewer's guidance, the proposed sentence was replaced as following:

Corticotrophin-releasing hormone (CRH) plays a key role in regulating the hypothalamic-pituitary-adrenal (HPA) axis.

Example: "Previous studies has been shown that formononetin exhibits significant estrogenic capabilities."

• Error: "Studies" is plural, so the verb should be "have."

• Correction: "Previous studies have shown that formononetin exhibits significant estrogenic capabilities."

Based on dear reviewer's guidance, the proposed sentence was replaced as following:

Previous studies have shown that formononetin exhibits significant estrogenic capabilities.

Example: "The results of Jang et al. showed that MCH plays an important role in pain modulation."

• Error: Mixing past and present tense.

• Correction: "The results of Jang et al. showed that MCH played an important role in pain modulation."

Based on dear reviewer's guidance, the proposed sentence was replaced as following:

The results of Jang et al. showed that MCH played an important role in pain modulation

The mean relative expression of orexin, Crh and Mch genes was measured using the RT-PCR method.

Example: "The mean relative expression of orexin, CRH, and MCH genes was measured using the RT-PCR method."

• Error: The comma before "and" is unnecessary in a simple list.

• Correction: "The mean relative expression of orexin, CRH and MCH genes was measured using the RT-PCR method."

Based on dear reviewer's guidance, the proposed sentence was replaced as following:

The mean relative expression of orexin, Crh and Mch genes was measured using the RT-PCR method.

Example: "Administration of 20 and 40 µg formononetin remarkably declined the mRNA levels of Crh, orexin and Mch in comparison to formalin group."

• Error: The phrase "in comparison to formalin group" is unclear.

• Correction: "Administration of 20 and 40 µg of formononetin significantly reduced the mRNA levels of Crh, orexin, and Mch compared to the formalin group."

Based on dear reviewer's guidance, the proposed sentence was replaced as following:

Administration of 20 and 40 µg of formononetin significantly reduced the mRNA levels of Crh, orexin, and Mch compared to the formalin group.

Example: "The animals were kept in individual cage to spend one-week recovery period."

• Error: "Individual cage" should be plural.

• Correction: "The animals were kept in individual cages to spend a one-week recovery period."

Based on dear reviewer's guidance, the proposed sentence was replaced as following:

The animals were kept in individual cages to spend a one-week recovery period.

Example: "The study involved twenty rats divided into four groups (n = 5); groups 1 and 2 had been the control group and the pain model group, which received saline."

• Error: Inconsistent structure in listing groups.

• Correction: "The study involved twenty rats divided into four groups (n = 5): Group 1 served as the control group, while Groups 2, 3, and 4 were the pain model groups receiving saline and formononetin, respectively."

Based on dear reviewer's guidance, the proposed sentence was replaced as following:

groups 1 and 2, respectively, had been the control group and the pain model group, that received saline. Groups 3 and 4 have been the pain model rats that received 20 and 40 µg of formononetin.

OPEN PEER REVIEW

Revision (1)

Sara Bahrainian : Associate Editor

23 Oct 2024

Thank you for submitting your valuable manuscript. The text is in need for some minor revisions. Please highlight any changes and submit a point to point response to the questions.

OPEN PEER REVIEW

Revision (2)

Reply to Reviewers

Ideally, the reviewing process can significantly improve the submitted manuscripts by allowing the authors to take into account the advice of reviewers. Author(s) must reply to all reviewers' comments in a separate Word file, point by point. A "**Reply to Reviewers**" document is submitted along with revised manuscript during submission of revised files, summarizing the changes that the authors made in response to the reviewers' comments. The responses to reviewers' comments specifies how the authors addressed each comment the reviewers made.

You can read the authors' responses to the reviewers' comments in the next page.

Dear Editor in- Chief

By special thanks of valuable comments of dear reviewers to improve the quality of the present manuscript, the responses were highlighted in the text. Also they were mentioned in the present file as following:

1-the number of rats in each group is too small to interpret the Formononetin mechanism of action in pain. Could the author add references that used a similar number of rat group sizes?

By thanks of valuable comment of reviewer, it should be mentioned that in experimental studies using animals, based on the limitation of research ethics committee, the small sample size (n = 5 or 6 per group) usually was chosen and accepted for each group. Some references with the sample size of 5-6 animals for each group in the experimental studies are mentioned as following:

1. Goto T, Marota J, Crosby G. Nitrous oxide induces preemptive analgesia in the rat that is antagonized by halothane Anesthesiology. 1994 Feb;80(2):409-16.
2. Mohammadpour MJ, Nourizadeh E, Mahmoudi F. Analgesic effect of trans-anethole via downregulation of hypothalamic orexin and melanin-concentrating hormone gene expression in the rat. Journal of Basic and Clinical Pathophysiology. 2023.
3. Matboli M, Helmy Hasanin A, Hamady S, Khairy E, Hussein Mohamed R, Abdul- Ela YM, Hussein Raafat M, Abdel Gawad Elsebay S, Emam HY, Shamekh R, Agwa SHA. Anti-inflammatory effect of trans-anethol in a rat model of myocardial ischemia-reperfusion injury. Biomed Pharmacother. 2022: 150:113070.
4. Cavendish RL, de Souza Santos J, Neto RB, Paixão AO, Oliveira JV, de Araujo ED, e Silva AA, Thomazzi SM, Cardoso JC, Gomes MZ. Antinociceptive and anti-inflammatory effects of Brazilian red propolis extract and formononetin in rodents. Journal of Ethnopharmacology. 2015 Sep 15;173: 127-33.
5. Bayrami A, Shirdel A, Rahim Pouran Sh, Mahmoudi F, Habibi-Yangjeh A, Singh R, Abdul Raman AA. Co-regulative effects of chitosan-fennel seed extract system on the hormonal and biochemical factors involved in the polycystic ovarian syndrome. Materials Science & Engineering C. 2020,
6. Mahmoudi F, Mahmoudi F, Haghighat Gollo Kh, Amini MM. Biosynthesis of novel silver nanoparticles using Eryngium thyrsoideum Boiss extract and comparison of their

antidiabetic activity with chemical synthesized silver nanoparticles in diabetic rats. Biological Trace Element Research. 2020, 127(1): 1-12.

7. Fan SH, Akbar Ali N, Fredalina Basri D. Evaluation of analgesic activity of the methanol xtract from the Galls of *Quercus infectoria* (Olivier) in rats. Evid Based Complement Alternat Med. 2014 Aug 31; 2014:976764.
8. Khazali H, Mahmoudi F. Morphine and kisspeptin influences on 5-α reductase and aromatase gene expression in adult male rats Iran J Basic Med Sci. 2019 Dec;22(12):1462-1467.

Also, the authors should use conventional analgesics as positive control alongside Formononetin for comparison. Why authors did not use positive control?

To evaluate the behavioral tests in the present study, in addition to formalin and formononetin groups, we also have two other groups which received saline and diclofenac **(**as positive control) respectively. But we did not report the results of the positive control in the text. Based on dear reviewer's suggestion if necessary we could substitute the results and figure in the text as following figure:

Saline injection into the animals' paws did not induce pain-related behavior. Based on the analysis of the data of the behavioral test, 20 µg formononetin caused a significant decrease in the pain score of both phases 1 and 2 compared to the formalin group **(**Figure 1, P≤0.05**).** Injection 40 µg formononetin remarkably reduced the pain score compared to the formalin group only in the phase 2**.** (Figure 1, P≤0.05). The injection of 20 µg diclofenac led to a significant reduction in the pain score of phases 1 and 2 compared to the formalin group (Figure 1, P≤0.05) However, diclofenac did not significantly decrease the pain score of phases 1 and 2 compared to of phases 1 and 2 of 20 and 40 µg formononetin (Figure 1).

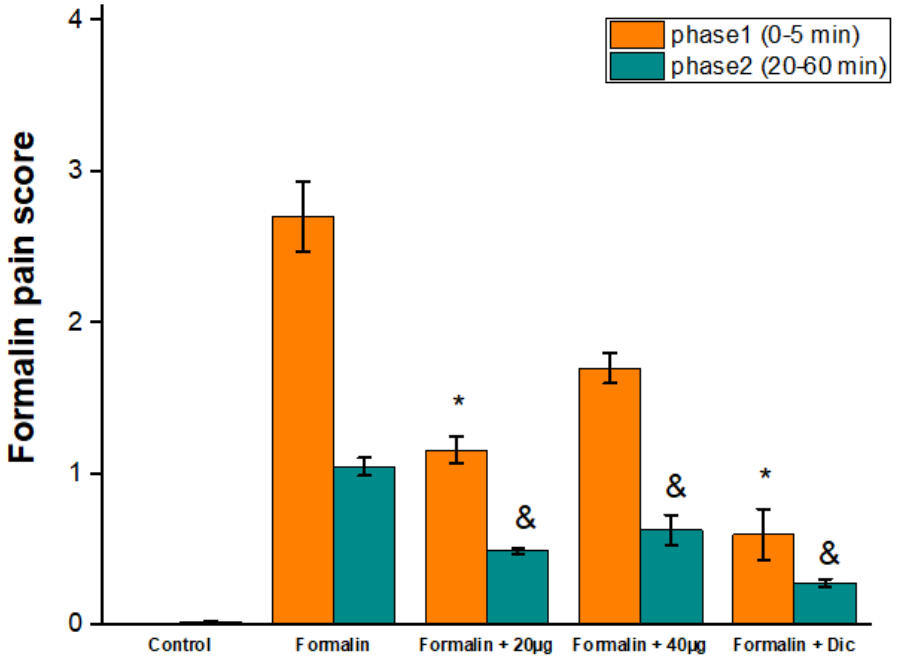


Figure 1. The effects of formononetin and diclofenac (Dic) on the pain score. Phase 1 (0-5 min and phase2 (20-60 min). *: compared with control(phase1); &: compared with control(phase2).

2-Please check the names of Mch and Crh similarly in the whole manuscript in the sections you did not mean Mch and Crh as the names of the gene. Also, in the title please use the entire name of Mch and Crh because they are mentioned for the first time if they mean signaling pathway

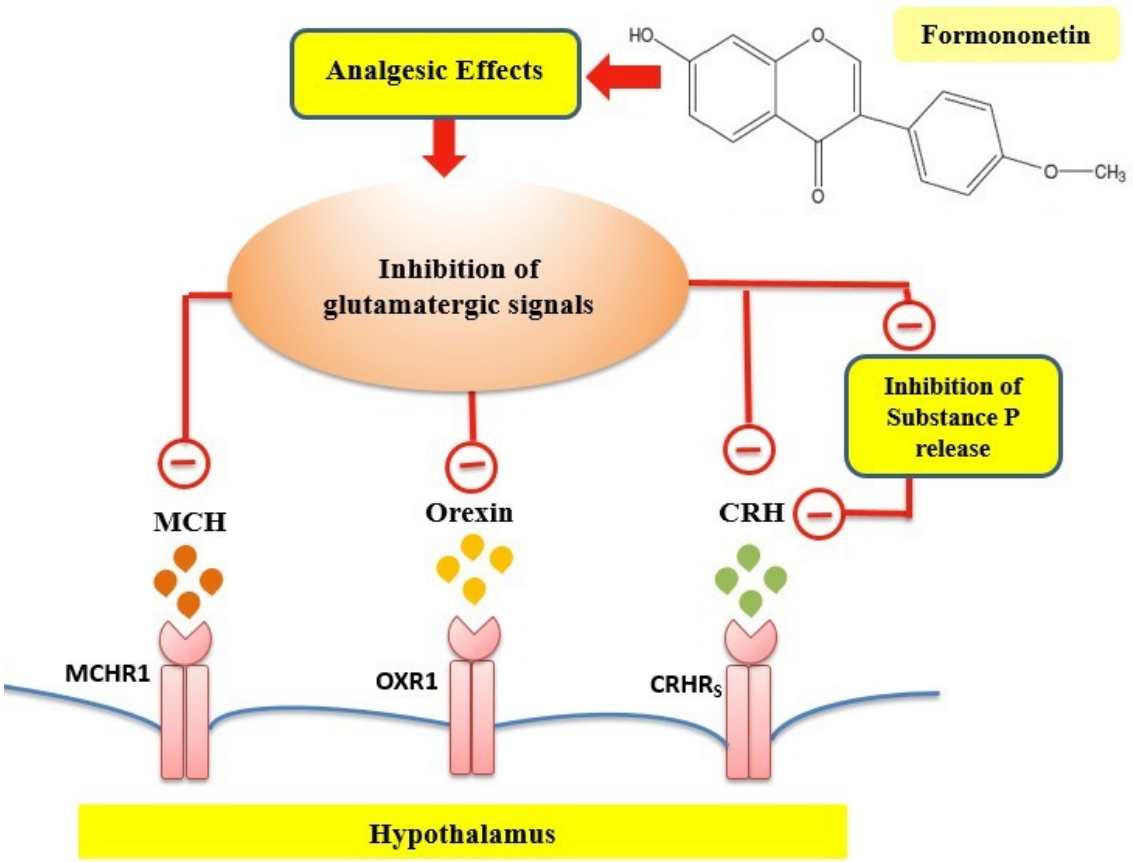
According to the dear reviewer's guidance, this items were edited in the title and the text.

3- Please check the keyword in the MESH. Using gene name of Mch or Crh is better or using the name of signaling pathway?

According to the suggestion of dear reviewer, the title was corrected as following: Analgesic effects of formononetin: An investigation of the role of hypothalamic corticotrophin releasing hormone, orexin, and melanin concentrating hormone signaling pathways in a pain model of rats

4-Please make the schematic picture (the network) of how Formononetin could affect the receptor and downstream genes to act as an analgesic.

Based on dear reviewer suggestion, schematic picture was prepared as following:



The hypothalamic putative pathways associated with the analgesic effects of formononetin.

5- Please use subtitle in the result section to separate each result section.

According to the suggestion of dear reviewer, subtitles was added to the results section as following:

4.1. Effects of formononetin on behavioral tests

4.2. Effects of formononetin on the hypothalamic mRNA levels of *Crh*, *Mch* and *Hcrt*

6- In line 91 the word “the” at the starting sentence should be used capital.

According to the dear reviewer's guidance, this item was edited in the text as following:

The animal's behavior was observed using a 45° angle mirror.

7-Please mention in the RT-PCR how many fold changes decrease the expression of orexin, Mch, and Crh compared to formalin.

Based on dear reviewer guidance, the amount of fold changes were added to results part as following:

The relative gene expression of *Crh* in the formalin-induced rats was significantly increased compared to the control group receiving saline (control:1±0.026; formalin group: 3.5±0.3) (Figure 4, P≤0.05). Administration of 20 and 40 µg formononetin significantly reduced the mRNA levels of *Crh* compared to the formalin group (20 µg formononetin receiving group: 1.85± 0.09; 40 µg formononetin receiving group: 1.1± 0.08) (Figure 2, P≤0.05).

When compared to the control group receiving saline, the formalin-induced pain model exhibited a substantial increase in *Hcrt* gene expression (control:1±0.023; formalin group: 3.02±0.19) (Figure 4, P≤0.05). The mean relative gene expression of the *Hcrt* was significantly lower in both groups receiving 20 and 40 µg formononetin than in the formalin group (20 µg formononetin receiving group: 0.77± .089; 40 µg formononetin receiving group: 0.32± 0.1) (Figure 3, P≤0.05).

In comparison to the control group receiving saline, the formalin-induced pain model group exhibited a considerable increase in *Mch* gene expression (control:1±0.02; formalin group: 3.7±0.32) (Figure 4, P≤0.05). The *Mch* gene expression was substantially lower in the groups receiving 20 and 40 µg formononetin than in the formalin- group (20 µg formononetin receiving group: 0.88± .05; 40 µg formononetin receiving group: 0.17± 0.06) (Figure 4, P≤0.05). Also, a remarkable decrease was occurred between the impacts of 20 and 40 µg formononetin (Figure 4, P≤0.05).

8-in the conclusion please mention the problem of this study and future perspective that could be down to proof analgesic effect of Formononetin.

Based on dear reviewer guidance, conclusion part was rewritten as following:

The present study demonstrates the analgesic potential of formononetin, in formalin- induced pain through modulating mRNA levels of hypothalamic *Hcrt*, *Crh* and *Mch*. Estrogenic effects of formonnetin may be involved in the down regulation of intra hypothalamic orexin and MCH neural signaling pathways upstream CRH neurons to relieve pain. One important limitation of the present study was the impossibility of using the western blot technique to detect the protein levels in samples due to the high cost of the materials. Further studies are needed to investigate the precise analgesic mechanisms of formononetin via determination the mRNA and protein levels of other nociception related neuropeptides such as substance P and dynorphin in hypothalamus and spinal cord. Also, to find some neural signaling pathways which through formononetin may exerts inhibitory effects on *Hcrt*, *Crh* and *Mch* and other neuropeptides, it is strongly suggested that future researches aimed to study the analgesic effects of formononetin by using the antagonist of glutamatergic, opioid, noradrenergic and GABAergic receptors in inflammatory, acute or neuropathic pain models.

OPEN PEER REVIEW

Revision (2)

REFEREE : Associate Editor

24 Nov 2024

Please revise the manuscript and submit a point-by-point response to the reviewers' comments and concerns and highlight any changes.
It is necessary that the manuscript be checked for grammar and English language before resubmission since this matter has been noted in previous revisions and not corrected.
Best regards

OPEN PEER REVIEW

Revision (3)

Reply to Reviewers

Ideally, the reviewing process can significantly improve the submitted manuscripts by allowing the authors to take into account the advice of reviewers. Author(s) must reply to all reviewers' comments in a separate Word file, point by point. A "**Reply to Reviewers**" document is submitted along with revised manuscript during submission of revised files, summarizing the changes that the authors made in response to the reviewers' comments. The responses to reviewers' comments specifies how the authors addressed each comment the reviewers made.

You can read the authors' responses to the reviewers' comments in the next page.

Dear Editor in- Chief
By special thanks of valuable comments of dear reviewers to improve the quality of the present manuscript, the responses were highlighted in the text. Also they were mentioned in the present file as following:

Reviewer 1:
1-Please check the whole manuscript grammatically.
Based on the guidance of dear reviewer, the whole manuscript checked grammatically.

2- Please use the full name of Hcrt, Crh, and Mch in the abstract in the objective part then the authors can use the abbreviation.
Based on the guidance of dear reviewer, full name of genes in abstract as following:
hypocretin (*Hcr*), *corticotrophin-releasing hormone* (*Crh)*, and *melanin-concentrating hormone (Mch).*

3-Please add the diclofenac result in the text for the pain score plot and mRNA expression of Hcrt, Crh, and Mch. Why did use diclofenac as a nonsteroidal anti-inflammatory drug instead of using an Analgesic drug as a positive control in this study? Please mention the reason in the text.
Based on the guidance of dear reviewer, the results of diclofenac were added to the text as following:
Based on several studies, the formalin- induced pain model, has two different phases and these two phases of the formalin test have different nociceptive mechanisms. The early phase is due to a direct activation of peripheral nociceptors. The late phase reflecting ongoing central sensitization and inflammatory response with inflammatory pain. The results of present study demonstrated that formononetin may able to affect both phase of formalin induced pain. Diclofenac is an NSAID that not only provides analgesic effects but also reduces inflammation. This dual action can be particularly useful in studies where both pain relief and reduction of inflammation are important outcomes.

Some of related references which used diclofenac as positive control are mentioned as following:
Hunskaar S, Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain. 1987 Jul 1;30(1):103-14.
Adibkia K, Nayebi AM, Barzegar-Jalali M, Hosseinzadeh S, Ghanbarzadeh S, Shiva A. Comparison of the analgesic effect of diclofenac Sodium-Eudragit® RS100 solid dispersion and nanoparticles using formalin test in the rats. Advanced pharmaceutical bulletin. 2015 Mar;5(1):77.
Mahmoud TM, Nafady MM, Farouk HO, Mahmoud DM, Ahmed YM, Zaki RM, Hamad DS. Novel bile salt stabilized vesicles-mediated effective topical delivery of diclofenac sodium: A new therapeutic approach for pain and inflammation. Pharmaceuticals. 2022 Sep 5;15(9):1106.

4-As the keywords of the article use the abbreviation of Hcrt, Crh, and Mch, did the authors check them in the MESH that used the full name or abbreviation of Hcrt, Crh, and Mch in the article?
Based on the guidance of dear reviewer, these words were checked in MeSH and corrected in the text as following:
hypocretin (Hcr), corticotrophin-releasing hormone (Crh), and melanin-concentrating hormone (Mch).

5-as the authors did not use an Analgesic drug as a positive control in this study please mention it as a limitation of this study in the conclusion.
Based on the guidance and suggestion of dear reviewer, this limitation was added to the text as following:
Results of the present trial warrant further dose responses studies in human and different pain models of animals such as fibromyalgia, inflammatory pain, spinal pain and sciatica to ensure the analgesic effects of formononetin without serious harmful consequences. Also, in the present study, diclofenac, a non-steroidal anti-inflammatory drug, was used as positive control to compare the pain relieving effects of formononetin. So, lack of using analgesic drugs may be one limitation and it is suggested that further studies aimed to compare the pain relieving effects of formononetin with the analgesic drugs such as morphine, codeine, ibuprofen and naproxen, pethidine.

.6-Please add the schematic pathways associated with the analgesic effects of formononetin in the manuscript.
Based on dear reviewer suggestion, schematic picture was prepared as following and added to text.

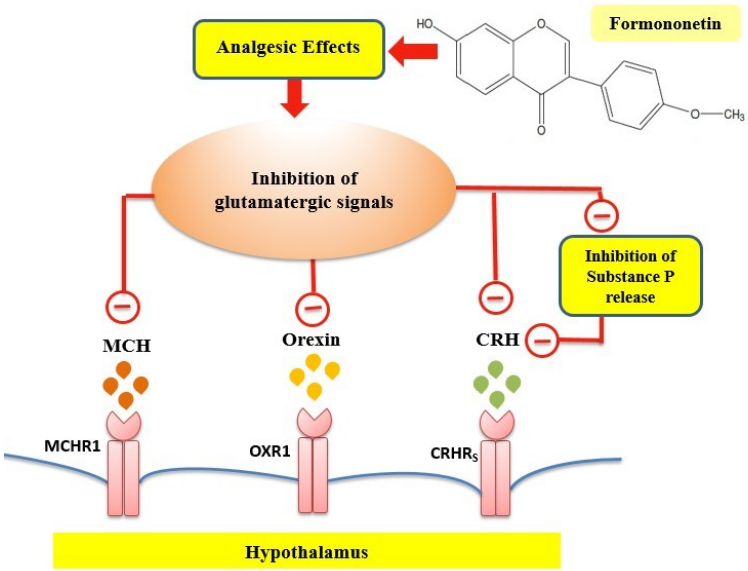


Figure 5. The hypothalamic putative neural pathways associated with the analgesic effects of formononetin.

7-This sentence “Therefore, it is assumed that formononetin, leads to a decrease in Crh and Hcrt gene expression by reducing the activity of α1-adrenoceptors in pain model of rats (9, 26, 37-41).” Should not be a last sentence in discussion. this sentence is the hypothesis of this study. In my opinion its better to mention that formononitin according to previous study could have effect on pain and in the current work the authors want to proof the mechanism of action in introduction section of article and in the discussion, authors compared the result of current work with studies which have been published previously.
Based on the guidance and suggestion of dear reviewer, this paragraph was corrected in discussion as following:

Reducing the activity of adrenergic signaling pathway may be another proposed mechanism for formononetin to decrease the hypothalamic *Crh* and *Hcrt* gene expression. A previous study demonstrated that formononetin reduces the expression of α1-adrenoceptors receptors. Also it has been established that α1-adrenoceptors expression increases on the orexin and CRH neurons during painful conditions, to activate the CRH and orexin neural signaling pathways. So, inhibitory effects of formononetin on adrenergic pathway may be a possible mechanism in the down- regulation of *Crh* and *Hcrt* gene expression to reduced pain.

Reviewer 2:

**Background

1. The background does an excellent job justifying why new analgesics are necessary. However, it could link more directly to the hypothalamus's role in pain modulation to strengthen the rationale for studying formononetin's hypothalamic effects.

Based on the suggestion of dear reviewer, hypothalamus's role in pain modulation was added to the text as following:

The hypothalamus (especially lateral hypothalamus and periventricular nuclei) is considered as an important central centers to modulate pain. It is established that hypothalamus receives inputs from multiple neural signaling pathway to integrate and coordinate the body's response to pain. In fact, induction and relieving of pain somewhat depends on the release of several pain-related peptides from the hypothalamus. In response to pain, the hypothalamus activates the sympathetic nervous system, leading to release of pain and stress related neuropeptides and hormones to modulate the perception and sensitivity of pain. Also, the hypothalamus is linked to the brain regions involved in the descending pain modulation pathways, such as the periaqueductal gray (PAG) which can inhibit or facilitate pain signals traveling from the spinal cord to higher brain centers (2-4).

Related references to the hypothalamus's role in pain modulation was added to the text as following:

Chao CC, Tseng MT, Hsieh PC, Lin CH, Huang SL, Hsieh ST, Chiang MC. Brain mechanisms of pain and dysautonomia in diabetic neuropathy: connectivity changes in thalamus and hypothalamus. The Journal of Clinical Endocrinology & Metabolism. 2022 Mar 1;107(3): e1167-80.

Jovanovic F, Jovanovic V, Knezevic NN. Glucocorticoid hormones as modulators of the kynurenine pathway in chronic pain conditions. Cells. 2023 Apr 18;12(8):1178.

de Git KC, van Tuijl DC, Luijendijk MC, Wolterink-Donselaar IG, Ghanem A, Conzelmann KK, Adan RA. Anatomical projections of the dorsomedial hypothalamus to the periaqueductal grey and their role in thermoregulation: a cautionary note. Physiological Reports. 2018 Jul;6(14): e13807.

2. The section includes references on formononetin's antioxidant and anti-inflammatory properties but lacks citations about its central effects, which would reinforce the study's relevance.

Intra hypothalamic molecular mechanism for analgesic effects of formononetin was not studied still and the present study for the first time try to investigate the effects of formononetin on hypothalamic pain related neuropeptides such as orexin, Crh and Mch. However, a number of previous studies, had reported the central positive effects of formononetin. Based on the dear reviewer suggestion a summary of these previous works were added to the text as following:

In the rat traumatic brain injury model, formononetin caused an increase in the cortical proliferation and reduction in serum IL-6 and TNF-α. Moreover, formononetin ameliorates neuro-inflammation in LPS-stimulated microglia by significantly reducing the production of TNF-α, IL-6, and IL-1β. Another study shows that formononetin reduces hippocampal neuro-inflammation and improves depressive-like behaviors in rat (11-13).

Related references to the positive effects of formononetin on brain was added to the text as following:

Singh L, Kaur H, Chandra Arya G, Bhatti R. Neuroprotective potential of formononetin, a naturally occurring isoflavone phytoestrogen. Chemical Biology & Drug Design. 2024 Jan;103(1): e14353.

El-Bakoush A, Olajide OA. Formononetin inhibits neuroinflammation and increases estrogen receptor beta (ERβ) protein expression in BV2 microglia. International immunopharmacology. 2018 Aug 1; 61:325-37.

Li M, Liu H, Peng S, Su P, Xu E, Bai M, Li Y. Effect of Formononetin on Lipopolysaccharide-Induced Depressive-Like Behaviors and Neuroinflammation in Mice. Chinese medicine and natural products. 2023 Sep;3(03): e126-32.

3. The mention of CRH, orexin, and MCH is informative but would benefit from explaining their specific roles in pain signaling to clarify why they were chosen for study.

Based on the dear reviewer suggestion their roles in pain signaling was added to the text as following:

The physiological function of the orexin in pain modulation has been confirmed by studying mechanical, chemical, thermal induced pain (15). Based on previous studies the activity of the orexin neurons increases and injection of orexin can alleviate pain in animal models of chronic neuropathic and inflammatory pain (16, 17). Also previous studies demonstrated that orexin neurons of the lateral hypothalamus co-localize with substance P afferents of dorsal root ganglion neurons, which further reinforces its confirmed role in pain regulation (18).

Based on the previous finding, during pain process, the CRH neurons of the paraventricular nucleus (PVN) of the hypothalamus are activated by pain- induced neurotransmitters such as glutamate and their activation in turn results to modulate the pain responses (20)

Also, based on documented studies, MCH neurons co- express glutamate and they are activated by glutamatergic signals during pain induction (23, 24).

4. While prior findings on formononetin are mentioned, providing more detail about its known or hypothesized effects on central pain modulation would add context.
Based on the dear reviewer suggestion previous central mechanisms related to analgesic effects of formononetin and also, a reason for the present study to select the orexin, MCH and CRH neural pathways in the mediating analgesic effects of formononetin were added to the text as following:
Analgesic effects of formononetin has been demonstrated based on behavioral tests in different pain models. However, few studies have been conducted to find central mechanisms mediating the analgesic effects of formononetin. So far, previous studies demonstrated the role of central GABAergic and glutamatergic signaling pathways in the damping of pain by formononetin. The GABA acts as pain relieving factor. However, glutamate activates several neural pathways in the brain to induce pain. As hypothalamic pain-related neuropeptides such as MCH, orexin, CRH receive direct inputs from GABAergic and glutamatergic neurons, the present study try to find the role of MCH, orexin and CRH neural pathways in mediating analgesic effect of formononetin on pain.

5. It is better to place the purpose of the study at the end of the introduction in a continuous manner and avoid placing the purpose of the study as a separate section
According to the suggestion of the dear reviewer, this item was corrected in the text.

**Methods and materials

1. Control Groups and Doses: While the doses of formononetin are given, a rationale for selecting 20 and 40 µg is missing, which is critical to demonstrate that these doses are therapeutically relevant.
To select the effective doses of drug, behavioal tests related to pain responses were used. So, at first rats in several groups received 5, 10, 20, 40 and 80 micrograms doses of the drug and between the used doses, based on behavioral responses we choose 20 and 40 micrograms formononetin to investigate the molecular mechanisms may mediate the anti- pain effects of formononetin. Also, choosing the effective dose were based on our previous study in stress model rats which these doses were able to affect hypothalamic CRH and MCH gene expression.

Based on dear reviewer guidance the related reference was added to the text as following:

Basirat E, Mahmoudi F, Khazali H. The expression of melanin concentrating hormone and corticotrophin releasing hormone genes in a stress model rats receiving formononetin. Gene Cell and Tissue. 2024. 11(4): 1-6

**Discussion

1. Comparison with Previous Studies: The discussion references previous studies but could more thoroughly discuss how this study's findings expand on or contrast with prior work.

Intra hypothalamic molecular mechanism for analgesic effects of formononetin was not studied still and the present study for the first time try to investigate the effects of formononetin on hypothalamic pain related neuropeptides such as orexin, CRH and MCH. So, direct comparing of the present result with previous ones was not possible for our discusson. However, a few number of previous studies, had reported the central positive effects of formononetin. So, based on the dear reviewer suggestion we try to expand and present some central mechanism may be involved in the effects of formononetin on CRH, MCH and orexin neural pathway as following:
Formononetin may downregulate Crh gene expression via interaction with GABAergic system. Inputs from GABAergic neurons in the lateral hypothalamus regulate CRH activity through an inhibitory mechanism. A previous study showed that Cajanus cajan which one of its main derivative is formononetin, activates the GABAA receptors. So, down regulation of the Crh gene by formononetin may be somewhat due to its influence on GABArgic neural pathways which in turn affects the activity of the CRH neurons (39, 40).
The related reference was added to the text as following:

KJ, M.I.K., 2002. GABAergic innervation of corticotrophin-releasing hormone (CRH)-secreting parvocellular neurons and its plasticity as demonstrated by quantitative immunoelectron microscopyNeuroscience113581-592. 28.
Miklos, IH, and Kovacs, KJ (2002). GABAergic innervation of corticotrophin-releasing hormone (CRH)-secreting parvocellular neurons and its plasticity as demonstrated by quantitative immunoelectron microscopy. *Neuroscience*, *113*, pp.581-592.
Olubodun-Obadun TG, Ishola IO, Adesokan TP, Anih BO, Adeyemi OO. Antidepressant-and anxiolytic-like actions of Cajanus cajan seed extract mediated through monoaminergic, nitric oxide-cyclic GMP and GABAergic pathways. Journal of Ethnopharmacology. 2023 Apr 24;306: 116142.

2. Mechanism Speculation: There is an interesting discussion on CRH and glutamate regulation; however, speculative mechanisms, such as GABAergic effects, could use more direct support from literature.
Based on dear reviewer guidance the GABAergic effects related to fomononetin effect was added to the text as following:

Formononetin may downregulate *Crh* gene expression via interaction with GABAergic system. Inputs from GABAergic neurons in the lateral hypothalamus regulate CRH activity through an inhibitory mechanism. A previous study showed that *Cajanus cajan* which one of its main derivative is formononetin, activates the $GABA_A$ receptors. So, down regulation of the *Crh* gene by formononetin may be somewhat due to its influence on GABArgic neural pathways which in turn affects the activity of the CRH neurons.
The related reference was added to the text as following:

KJ, M.I.K., 2002. GABAergic innervation of corticotrophin-releasing hormone (CRH)-secreting parvocellular neurons and its plasticity as demonstrated by quantitative immunoelectron microscopyNeuroscience113581-592. 28.

Miklos, IH, and Kovacs, KJ (2002). GABAergic innervation of corticotrophin-releasing hormone (CRH)-secreting parvocellular neurons and its plasticity as demonstrated by quantitative immunoelectron microscopy. *Neuroscience*, *113*, pp.581-592. Olubodun-Obadun TG, Ishola IO, Adesokan TP, Anih BO, Adeyemi OO. Antidepressant-and anxiolytic-like actions of Cajanus cajan seed extract mediated through monoaminergic, nitric oxide-cyclic GMP and GABAergic pathways. Journal of Ethnopharmacology. 2023 Apr 24;306: 116142.

3. Limitations: This section could be improved by addressing limitations, such as the small sample size and the use of a single animal model, which would enhance the study's transparency.

Based on the guidance and suggestion of dear reviewer, limitation was improved in the text as following:

Results of the present trial warrant further dose responses studies in human and different pain models of animals such as fibromyalgia, inflammatory pain, spinal pain and sciatica to ensure the analgesic effects of formononetin without serious harmful consequences. Also, in the present study, diclofenac, a non-steroidal anti-inflammatory drug, was used as positive control to compare the pain relieving effects of formononetin. So, lack of using analgesic drugs may be one limitation and it is suggested that further studies aimed to compare the pain relieving effects of formononetin with the analgesic drugs such as morphine, codeine, ibuprofen and naproxen, pethidine.

4. Implications of Findings: The discussion could better explore the potential clinical implications of formononetin’s effects on pain modulation or suggest future studies to test its impact on diverse pain models.

Based on the present results formononetin could be considers as a potential target for pain management. Formononetin may be suggested as a natural alternative to conventional painkillers, potentially reducing opioid dependence and minimizing associated side effects. Its ability to modulate estrogen receptors may provide targeted pain relief for conditions affected by hormonal fluctuations. The anti-inflammatory effects of formononetin can be useful in the treatment of chronic pain conditions characterized by inflammation, such as arthritis or fibromyalgia. Formononetin may be considered as part of combination therapies, increasing the effectiveness of existing treatments while reducing doses and side effects. However, results of the present trial warrant further studies in human and different pain models of animals such as fibromyalgia, inflammatory pain, spinal pain and sciatica to ensure the analgesic effects of formononetin without serious harmful consequences.

**Conclusion

1. Limitations Mentioned in Conclusion: Acknowledging significant limitations here would improve transparency, as many readers may only review the conclusion.

Based on the guidance and suggestion of dear reviewer, limitation was added to the conclusion as following:

Results of the present trial warrant further dose responses studies in human and different pain models of animals such as fibromyalgia, inflammatory pain, spinal pain and sciatica to ensure the analgesic effects of formononetin without serious harmful consequences. Also, in the present study, diclofenac, a non-steroidal anti-inflammatory drug, was used as positive control to compare the pain relieving effects of formononetin. So, lack of using analgesic

drugs may be one limitation and it is suggested that further studies aimed to compare the pain relieving effects of formononetin with the analgesic drugs such as morphine, codeine, ibuprofen and naproxen, pethidine.

2. specific follow-up experiments, such as dose-response studies or testing in different pain models, would be suggested to strengthen the conclusion.
Based on the guidance dear reviewer, this valuable suggestion was added to the conclusion as following:

Results of the present trial warrant further dose responses studies in human and different pain models of animals such as fibromyalgia, inflammatory pain, spinal pain and sciatica to ensure the analgesic effects of formononetin without serious harmful consequences.

OPEN PEER REVIEW

Revision (3)

Sara Bahrainian : Associate Editor

24 Dec 2024

Thank you for submitting your valuable manuscript to Jundishapur Journal of Natural Pharmaceutical products.
I am glad to announce that your manuscript has been accepted for publishing in this journal.

Best Regards